Mitochondrial Permeability Transition Pore Assay Kit

Introduction

Mitochondria are the energy centers of cells and play an important role in many cellular processes. The Mitochondrial Permeability Transition Pore (MPTP) is a channel on mitochondria composed of inner and outer mitochondria membranes that participates in the release of mitochondrial substances during cell death such as apoptosis. Therefore, the opening of MPTP is considered an important marker of cell death such as apoptosis.

The Mitochondrial Permeability Transition Pore Assay Kit is a kit that detects mitochondrial permeability pore opening by the fluorescent probe calcein AM. Calcein AM is a probe that can label living cells, which easily enters the cell and is hydrolyzed into green-fluorescent calcein by esterases. $CoCl_2$ can bind to calcein and quench the fluorescence of calcein, but the mitochondrial MPTP is normally turned off, so the green fluorescence of the mitochondrial calcein is retained. However, when Ca^{2+} ionophore ionomycin is further used to induce extracellular Ca^{2+} into the cell to trigger MPTP opening, then the green fluorescence of the mitochondrial calcein is quenched. This allows the degree of openness of mitochondrial MPTP to be judged by the intensity of green fluorescence in the mitochondria.

If the uncoupling agent CCCP is used instead of Ca^{2+} ionophore ionomycin, although the mitochondrial membrane potential is lost, MPTP does not open in a short period of time. So the fluorescence of the mitochondrial calcein does not weaken.

For flow cytometry, this kit is sufficient for 100 assays; for 96-well plates, this kit is sufficient for 1000 assays.

Components and Storage

Components	K2061-100 T
Calcein AM（1000X）	110 μL
$CoCl_2$（100X）	1 mL
Ionomycin（200X）	600 μL
Dilution buffer	200 mL
Cosolvent buffer（100X）	1.2 mL

This kit should be stored at -20°C, stable for 1 year. Calcein AM (1000X) and Ionomycin（200X）should be stored at -20°C away from light, and avoid repeated freeze/thaw cycles.

Protocol

1. Reagent preparation:

1) **Preparation of Calcein AM working solution:** Dilute 10 μL Calcein AM (1000X) and 100 μL Cosolvent buffer (100X) per 10 mL dilution buffer, and mix well to obtain Calcein AM working solution. The working solution is unstable, any unused working solution should be discarded after use.

***Note:** Calcein AM (1000X) is susceptible to hydrolysis. So, it is recommended to be prepared in single-use aliquots. The cosolvent buffer can increase the solubility of Calcein AM, and reduce precipitation in the dilution buffer. However, the cosolvent buffer is not necessary and cannot be used in special cases. The optimal concentration of Calcein AM working solution varies depending on the type of cells.

2) **Preparation of fluorescence quenching working solution:** Dilute appropriate $CoCl_2$ (100X) in Calcein AM working solution at a ratio of 1:100, and mix well to obtain fluorescence quenching working solution.

***Note:** The optimal concentration of fluorescence quenching working solution varies depending on the type of cells.

3) **Preparation of Ionomycin working solution:** Dilute appropriate Ionomycin (200X) in fluorescence quenching working solution at a ratio of 1:200, and mix well to obtain Ionomycin working solution.

***Note:** Ionomycin can form complex with Calcein, resulting in precipitation. So, the working solution needs to be prepared quickly to avoid precipitation. The optimal concentration of Ionomycin working solution varies depending on the type of cells.

4) **Preparation of CCCP working solution (optional):** Dilute appropriate CCCP (10 mM) in fluorescence quenching working solution at a ratio of 1:1000, and mix well to obtain CCCP working solution (10 μM).

***Note:** This kit does not provide CCCP. If needed, please purchase it separately (Catalog number: B5003).

2. Fluorescence microscopy detection:

Here, we take adherent cells as an example. For suspension cells, harvest cells and resuspend them in the correspondent working solution, then perform similarly to the adherent cells.

1) **Cell seeding:** Cells are seeded in 96-well plates or other plates, and cells can be treated as designed.

2) **Wash:** Remove the cell culture medium and wash cells in PBS one time.

***Note:** Serum and phenol red can increase background fluorescence. This step is to reduce the background caused by residual serum and phenol red.

3) **Staining:** Add appropriate Calcein AM working solution, fluorescence quenching working solution, Ionomycin working solution or CCCP working solution (optional) to cover cells. Incubate at 37°C away from light for 30-45 min. For 96-well plates, 100 μL per well working solution is needed. For 6-well plates, 1 mL per well working solution is needed.

***Note:** The optimal time for incubation varies depending on the type of cells.

4) **Incubation:** After incubation, replace the working solution with a fresh, pre-warmed medium. Incubate at 37°C away from light for 30 min to allow complete de-esterification of AM eaters.

5) **Detection:** After incubation, wash the cells with PBS 1-2 times. The fluorescence signal can be detected by a fluorescent microplate reader (Ex/Em: 494/517 nm). Or use a fluorescence microscope for

observation. Other counterstains can be continued if needed.

3. **Flow cytometry detection:**

   1) **Prepare cells:** For adherent cells, use trypsin to digest cells and then resuspend cells in culture medium. Washed cells in PBS one time. For suspension cells, centrifuge at 800 rpm for 5 min and wash with PBS one time.

   2) **Staining:** Add Calcein AM working solution, fluorescence quenching working solution, Ionomycin working solution or CCCP working solution (optional) to resuspend the cells pellet and adjust the cell density to 10^6 cells/mL. Incubate at 37°C away from light for 30 min. Samples only containing dilution buffer need to be prepared as negative controls for flow cytometry.

*Note: The optimal time for incubation varies depending on the type of cells.

   3) **Detection:** After incubation, wash cells in dilution buffer 1-2 times. Then resuspend cells with appropriate dilution buffer for flow cytometry. Other counterstaining can also be performed if needed.

*Note: After staining, the samples need to be placed on ice and detected within 1 h as much as possible.

Note

1. Calcein AM (1000X) is susceptible to hydrolysis. So, it is recommended to be prepared in single-use aliquots. Before use, allow the Calcein AM (1000X) to warm to room temperature.
2. Fluorescent probes are easy to quench, please protect them from light during storage and use.
3. $CoCl_2$ is corrosive and toxic, please pay attention to protection during experiments to avoid inhalation or direct contact with the human body. At the same time, $CoCl_2$ cannot be directly discharged into the environment.
4. For your safety and health, please wear lab coats and gloves during the experiment.
5. For research use only. Not to be used in clinical diagnostic or clinical trials.

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